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Total potentially available nucleosides of human milk by stage of lactation[1–4]

James L Leach, Jeffrey H Baxter, Bruce E Molitor, Mary B Ramstack, and Marc L Masor

ABSTRACT Human milk-borne ribonucleotides reportedly have important physiological roles in breast-fed infants. Previous studies measured the free nucleotide content of human milk. To more fully evaluate the physiological capacity of nucleotides in human milk, we determined the monomeric and polymeric ribonucleotide and ribonucleoside content of milk pooled from 11 American women. Subsequently, we determined the total potentially available nucleosides (TPAN) of pooled and individual milk samples segregated by stage of lactation from 100 women in three European countries to test for effect of culture and diet. The methodology simulated in vivo digestion. Polymeric ribonucleotide (primarily RNA), monomeric ribonucleotide, and ribonucleoside-containing adducts (eg, uridine diphosphate hexose) were enzymatically hydrolyzed to their constituent ribonucleosides, the preferred form for absorption. Free and enzymatically liberated nucleosides were then measured by HPLC to yield the TPAN value. The mean (± SD) TPAN concentration of the 16 pooled European samples, derived from the 100 individual samples, was 189 ± 70 μmol nucleoside/L human milk (range 82–402 μmol/L). The means (μmol/L human milk) of each nucleoside were 38 for uridine, 88 for cytidine, 31 for guanosine, and 32 for adenosine. These values included the contribution from the cellular portion of human milk. Only one of the 16 pooled samples contained a measurable amount of inosine (4 μmol/L). The potentially available ribonucleosides in the human milk samples were predominantly present as monomeric (36 ± 10%) and polymeric (48 ± 8%) nucleotides. This study demonstrates that the traditional measurement of the free nucleotide content of human milk (which accounts for neither polymeric nor cellular nucleotides) underestimates the total nucleotides available to the infant by ≥ 50%. *Am J Clin Nutr* 1995;61:1224–30.

KEY WORDS Human milk, ribonucleic acid, nucleotide, nucleoside, ribonucleoside

INTRODUCTION

Nucleotides are ubiquitous, low-molecular-weight compounds consisting of a nitrogenous base (usually adenine, cytosine, guanine, thymine, or uracil), a sugar moiety (ribose or deoxyribose), and one to three phosphate groups (1). They are essential in energy metabolism and enzymatic reactions and are the monomeric units of polymeric RNA and DNA (1). As second messengers (cAMP, cGMP) and components of cofactors (NAD, NADP, FAD), nucleotides are an integral part of carbohydrate, lipid, protein, and nucleic acid metabolism (1, 2).

Nucleotide concentrations are maintained by de novo synthesis and by a salvage pathway that recovers metabolized nucleotides and nucleosides originating from the diet or intermediary metabolism (1, 3). The two pathways are regulated by dietary availability to maintain an adequate and continuous supply of tissue nucleotides (4–6). Polymeric forms of nucleotides (DNA and RNA) are generally the primary dietary source of nucleotides (3). Polymeric nucleotides are digested by phosphodiesterases (ribonucleases and deoxyribonucleases) to nucleotides (3), which are further degraded by phosphatases to nucleosides, the preferred form for absorption in the small intestine (3, 7).

When metabolic demand exceeds the capacity for de novo synthesis, for instance, during periods of rapid growth or after injury, dietary nucleosides and nucleotides may become conditionally essential nutrients. Tests of this hypothesis in animal models have focused on tissues undergoing high rates of cellular proliferation or rapid growth, particularly the developing gut and the responsive immune system. Dietary nucleosides were reported to be important in the growth and maturation of the developing gut and to play several roles in immune function (8, 9). These roles include availability to lymphocytes unable to synthesize nucleotides (10), immune stimulation in mice when added to nucleotide-free diets (11, 12), improved response to sepsis in mice (13, 14), enhanced lymphocyte proliferation (15), stimulation of immunoglobulin production in peripheral lymphocytes (16), and increased natural killer cell activity (17).

The presence of ribonucleotides in human milk has prompted clinical research into their potential benefit for developing infants and has led to speculation as to whether they should be added to infant formula (18). The effect of dietary nucleotides on infant growth was first reported in 1963 (19). Subsequently, nucleotide supplementation reportedly altered the profile of plasma lipids and lipoproteins (20–23) and the fecal microflora (24) of formula-fed infants to be more like those of breast-fed infants; some of these claimed effects were not corroborated in other studies (25, 26). In another report, lymphocytes from infants fed nucleotide-fortified formula showed increased

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natural killer cell activity in an in vitro assay (27). Recently, nucleotide-fortified infant formula decreased the incidence of diarrhea in a group of infants of low socioeconomic status in Chile (28).

An accurate determination of the concentration and forms of ribonucleic acids in human milk is essential to evaluate their effect on outcomes of interest. Previous measurements have been nonspecific (29), or have measured only a portion of the total ribonucleic acid fraction (30, 31). Nonetheless, these data are the basis for the amount of ribonucleotide fortification in several commercial infant formulas. Because the entire polymeric ribonucleotide content of human milk has not been accurately measured, it has not been included as part of the nucleotide fortification of infant formula.

The method presented here measures all major sources of ribonucleotide in human milk potentially available for absorption and metabolism as ribonucleoside. It was first developed with a pooled, frozen sample of human milk from American women. Free nucleosides as well as those derived from nucleotides and nucleotide polymers were determined. Subsequently, several questions arose. Human milk is known to contain a significant number of cells; did the freezing and handling of the sample rupture these cells and release their nucleotide content? What was the contribution from nucleoside-containing adducts, such as uridine diphosphate glucose? Would the nucleotide concentration differ in the milk of women from other countries with diverse cultures and diets?

The present study was designed to answer these questions with an expanded sample size. Because all research on metabolically active nucleotides has been restricted to ribose-containing forms, deoxyribose forms were not considered. Henceforth, the terms nucleoside and nucleotide will refer only to ribose-containing forms.

SUBJECTS AND METHODS

For the initial method development, 11 lactating American women between 1 and 4 mo postpartum were brought to a collection center, where each completely emptied one breast under sterile conditions. These samples were immediately pooled, thoroughly mixed, divided into 10-mL aliquots, and frozen (−70 °C) until analyzed. Details of the analysis are essentially the same as those of the present study described below, except that measurement of nucleotide-containing adducts was not included.

We attempted to distinguish between cellular and noncellular pools of nucleotides during the development of the method described below using the initial sample of human milk. The concentration of polymeric and monomeric nucleotide and free nucleoside was measured in a previously frozen, deactivated, pooled human milk sample as described below. The same sample was reanalyzed after cellular disruption by high-intensity sonification (± 0.1%) (Triton X-100; Sigma, St Louis) before enzymatic hydrolysis. There was no significant increase in the concentration of polymeric and monomeric nucleotide and free nucleoside after the sonification procedure. Therefore, the measurement of the pooled and individual samples from the European human milk samples described below includes, and does not differentiate between, the cellular and noncellular pools of nucleic acids.

Subject selection

Human milk samples were collected from two sites in Italy, one in France and one in Germany. Sites were selected in these countries to address the question of the influence of differing diets and cultures on the total potentially available nucleosides (TPAN) of human milk. Subjects were selected from four stages of lactation: colostrum (through 2 d postpartum), transitional milk (3–10 d postpartum), early mature milk (1 mo postpartum), and late mature milk (3 mo postpartum). Five to seven individuals per site, per stage of lactation, contributed samples of human milk. A total of 100 individual samples were collected. Potential sample donors were contacted and the complete nature of the study described. If the potential donor expressed a willingness to participate in the study, written informed consent was obtained before collection of the sample.

Sample donors had no history of alcohol or drug abuse and had no medical condition or obstetrical complications thought to influence lactation. Donors had a singleton birth; had a hiatus of ≥ 15 mo since the cessation of breast-feeding a older child; had a preconceptional weight-for-height betwee 100% and 115% of ideal values; experienced adequate weigh gain throughout pregnancy, as determined by the investigato and gave birth after a gestation of > 36 wk. Donors were nc receiving any medication known to interfere with lactation an exclusively breast-fed their infant, ie, the infant was fed ≤ 12 mL formula/d.

Sample collection

Milk was expressed at a collection center. The mothe breast-fed the baby at midday on the same breast as the han dominance of the mother (ie, right breast for right-hande mothers). When the baby was satisfied, the mother applied a electric breast pump to the nursed breast to ensure complet emptying. Any milk collected was discarded. About 60–90 mi later, the mother washed the same breast with a mild soap an rinsed the breast repeatedly with distilled water. The breas pump was applied for ≈8 min to collect the sample while th baby suckled on the other breast to initiate let-down.

If a 50-mL sample could not be obtained in 8 min, the firs sample was immediately frozen and a second 8-min sampl was collected. Samples were collected into polyethylene con tainers labeled to indicate the donor's stage of lactation an stored at −75 °C. The frozen samples were stored at th collection site until all of the samples from that site had been collected. Frozen samples were then shipped for analysis in dry ice to the laboratory via overnight express delivery.

Sample pooling and deactivation

Application of the complete analytical scheme (four hydrolyses in duplicate) on 100 samples would have required 800 separate analyses. Because of the length of the procedure, a decision was made to pool samples at each site. The acceptability of this decision was tested by comparing 20 individual samples from one site (five at each stage of lactation) to pools of those samples at each stage of lactation. Human milk contains enzymes that can degrade nucleic acids. Treatment of the milk with strong base inactivates most interfering enzymes but does not alter concentrations of TPAN. Samples were held at −75 °C until analyzed. Samples from a single site were quickly thawed to room temperature, and aliquots of individual

samples at each stage of lactation were thoroughly mixed to provide a 20-mL sample of pooled milk. Sodium hydroxide (1 mol/L) was added (40 mL) and the samples were covered and stirred for 30–60 min. The pH of the samples was then adjusted to 7.0–7.5 with hydrochloric acid, and diluted to 100 mL with water. Five-milliliter aliquots of individual samples from one site were similarly treated (by using 10 mL sodium hydroxide and brought to 25 mL with water).

Enzymatic hydrolyses

Four distinct sample preparations were carried out in duplicate with 5 mL deactivated, diluted sample, stirred in a Pierce heating-stirring module (Reacti-Therm; Rockford, IL). An internal standard, 5-methylcytidine (30 μg; Sigma #M-6254) was added to every sample preparation. **Figure 1** depicts the action of the three enzymes used in these preparations. In preparation 1, duplicate deactivated human milk samples were incubated 16–18 h with nuclease (nuclease P1 phosphodiesterase, 19 U; Sigma #N-8630) to hydrolyze polymeric to monomeric nucleotide by using a modification of the procedure described by Gehrke and Kuo (32). This was followed by incubation with pyrophosphatase (nucleotide pyrophosphatase, 0.4 U; Sigma #P-7383) to release nucleotide from adducts, and with phosphatase (bacterial alkaline phosphatase, 16 U; Sigma #P-4252) to hydrolyze nucleotide to nucleoside by using the modified procedure of Gehrke and Kuo (32) for 3 h at 37 °C. Preparation 2 included both the nuclease and phosphatase hydrolysis, preparation 3 used the phosphatase hydrolysis only, and preparation 4 was the unhydrolyzed deactivated sample. Samples were then quantitatively transferred to 25-mL volumetric flasks with 12.5 mL 0.5 mol potassium phosphate/L, pH 10.5, and brought to the desired volume with water.

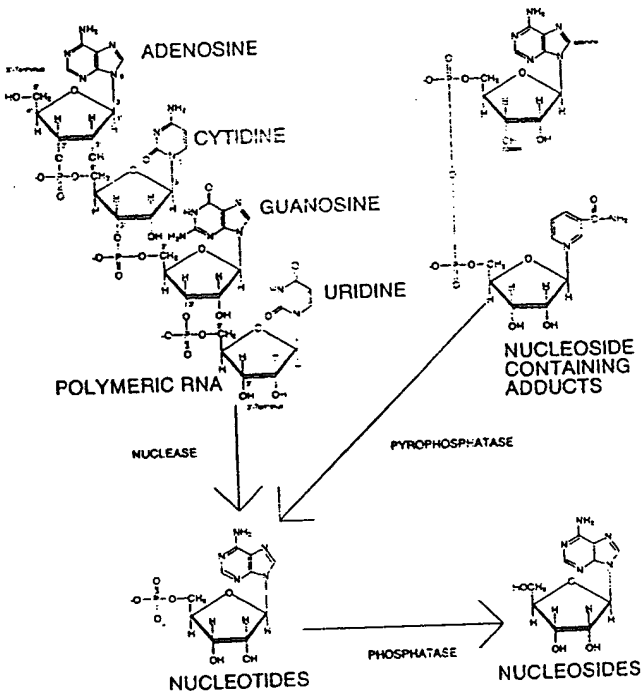

FIGURE 1. The enzymatic digestion of ribonucleotide. Polymeric RNA, and nucleoside-containing adducts (nucleoside-phosphate-phosphate-X, where X is any of a group of biologically relevant moieties, eg, NAD, UDP-glucose) are hydrolyzed to their corresponding nucleotides by the actions of nucleases and pyrophosphatases, respectively. Ribonucleotide is further hydrolyzed to nucleoside, the preferred form for absorption in the gut, by the action of phosphatases.

Solid-phase extraction

The procedure described here is based on the work of Uziel et al (33). The solid-phase extraction media was Affi-Gel 601 (#153-6101; Bio-Rad, Melville, NY). Hydrated, settled gel (150 μL) in a polypropylene microcentrifuge tube was washed twice with buffer (250 mmol potassium phosphate/L, pH 10.5) by vortexing, followed by centrifugation and removal of supernate. The washing procedure converted the gel to the basic form. A 1-mL aliquot sample from the enzymatic preparations described above was added to the gel and vortexed, binding the nucleosides to the gel. Contaminating compounds were removed from the gel-bound nucleosides by two 1-mL washings with the potassium phosphate buffer. Nucleosides were then eluted from the gel by using 750 μL 1 mol phosphoric acid/L and passed through a 0.22-μm filter directly into a vi.. - HPLC analysis.

HPLC analysis

The nucleosides were separated via reversed-phase, pairing chromatography on an octydecylsilane stationary column (#4M, 5314; Jones Chromatography, Lakewood, The mobile phase was 100 mmol potassium acetate/L, pH and 2 mmol hexane sulfonic acid/L (Sigma #H9026) organic modifier was acetonitrile, with an initial concent of 1%, which was linearly increased to 7% from 0 to 8 held at 7% for 2 min, and reequilibrated at 1% for 8 min t the next injection. The nucleosides were detected by a bance at 255 nm. Calibration curves for each nucleoside constructed by calculating the ratios of the area respon known concentrations of serially diluted nucleoside sta materials (Sigma products: U-3750, C-9505, I-4125, G- and A-9251) to the area of a fixed concentration of the in standard 5-methylcytidine. Linear regression gave corre coefficients > 0.9995. The sample concentration of eac cleoside was calculated by using the linear regression fo nucleoside and the area ratio (nucleoside-internal sta from analyses.

Data reduction and precision

The nucleoside concentrations measured after the four distinct sample preparations permitted determination of the amount of each nucleoside found in each form and the amount each form contributed to TPAN.

Preparation 1: Measurement of inherent free nucleosides and nucleosides resulting from nuclease, pyrophosphatase, and phosphatase hydrolysis gave the TPAN value.

Preparation 2: Hydrolysis with nuclease and phosphatase gave the amount of inherent, nucleotide-derived, and polymer-derived nucleosides. Preparation 1 − preparation 2 = nucleosides derived from nucleoside-containing adducts.

Preparation 3: Hydrolysis with phosphatase provided inherent and nucleotide-derived nucleosides. Preparation 2 − preparation 3 = polymer-derived nucleoside.

Preparation 4: No enzymatic hydrolysis yielded inherent nucleosides. Preparation 3 − preparation 4 = nucleotide-derived nucleoside.

Preliminary computations demonstrated that the SD was proportional to the mean. Therefore, the percent relative SD (% RSD) for each duplicate was a more appropriate estimate of the variability of the measurement than the SD.

Overfortification recoveries

An aqueous TPAN-fortified solution was prepared that contained ribonucleosides, 5′-mononucleotides, polymeric yeast RNA, and nucleoside-containing adducts (uridine diphosphate glucose, cytidine diphosphate choline, guanosine diphosphate mannose, and NAD), all at concentrations 100 times those typical for human milk. An aliquot was diluted 1:100 with water and hydrolyzed for 16 h in 0.2 mol potassium hydroxide/L to quantitatively cleave all of the polymeric RNA to 2′- and 3′-mononucleotides. The pH of this solution was adjusted to ≈9 with hydrochloric acid and incubated with alkaline phosphatase and nucleotide pyrophosphatase to hydrolyze all nucleotide and nucleoside-containing adducts to nucleoside, and the nucleoside concentrations were measured directly (without solid-phase extraction). Because previous work (data not shown) demonstrated that the phosphatase- and pyrophosphatase-catalyzed reactions were quantitative, this alkaline and enzymatic hydrolysis and HPLC analysis was used to define theoretical concentrations. The TPAN-fortified solution was diluted 1:100 with one of the pooled human milk samples (early mature milk from site 3, Italy) and the TPAN analysis carried out to determine recovery.

RESULTS

When the difference between the fortified and unfortified pooled sample is compared with the concentration derived from the alkaline enzymatic hydrolysis of the fortified solution, 91% of the theoretical TPAN value was recovered (Table 1). The method recovered within 5% of the "actual" value for cytidine and adenosine and underestimated the uridine value by ≈12% and the guanosine value by ≈24%. The %RSD of the measurement of each of the four nucleosides and the TPAN value is an indicator of the precision of the measurement (Table 1). The variance of the method makes only a very small contribution to the between-sample variance in this study.

Table 2 provides a summary of all TPAN data by site and by stage of lactation. Comparison between sites at each stage of lactation shows considerable variability. The mean ranges of TPAN values (μmol/L) from the different sites were 82–164 (colostrum), 144–210 (transitional milk), 172–402 (early mature milk), and 156–259 (late mature milk). Comparison between stages of lactation at each site shows equal variability in TPAN (μmol/L): 146–172 at site 1, 82–219 at site 2, 164–214 at site 3, and 150–402 at site 4. The mean TPAN (sites combined) was lowest in colostrum (137 μmol/L) but showed no consistent upward or downward trend in transitional milk (177 μmol/L), early mature milk (240 μmol/L), or late mature milk (202 μmol/L). Also shown in Table 2, the mean TPAN (excluding adducts) from pooled American milk (161 μmol/L) was within the range of the European milk (82–402 μmol/L).

The percentage of each form of the mean TPAN for the entire European pool is provided in Table 3. Most of the TPAN was present as polymeric (48 ± 8%; $\bar{x}$ ± SD) and monomeric

TABLE 1
Accuracy and precision of the total potentially available nucleosides (TPAN) method

Test samples	Uridine	Cytidine	Guanosine	Adenosine	TPAN
TPAN-fortified (μmol/L)[1]	64	70	73	69	276
Pooled milk (μmol/L)	67	146	91	97	402
Pooled milk + TPAN-fortified (μmol/L)	124	219	147	165	654
Difference (μmol/L)	57	73	55	67	252
Percent recovery (%)[2]	88	104	76	98	91
Precision of TPAN method					
Relative standard deviation (%RSD)[3]	3.6	2.0	2.0	2.0	1.9

[1] An aqueous solution of nucleosides, monomeric and polymeric nucleotides, and nucleoside-containing adducts at concentrations found in human milk, and subjected to alkaline and enzymatic hydrolysis to yield theoretically accurate concentrations.

[2] Difference between the fortified and unfortified pooled milk sample divided by the concentrations measured in the TPAN-fortified solution.

[3] Computed from 16 degree-of-freedom estimate of the variance.

TABLE 2
Nucleotide and total potentially available nucleoside (TPAN) in pooled human milk by stage of lactation[1]

	Uridine	Cytidine	Guanosine	Adenosine	TPAN
			μmol/L		
Colostrum					
Site 1	27	84	22	20	153
Site 2	21	33	15	13	82
Site 3	30	82	26	26	164
Site 4	24	84	20	22	150
Mean	26	71	21	21	137
Transitional milk					
Site 1	23	82	22	19	146
Site 2	33	76	19	17	144
Site 3	37	84	43	42	206
Site 4	36	100	36	38	210
Mean	32	86	30	29	177
Early mature milk					
Site 1	30	86	28	28	172
Site 2	50	79	23	21	173
Site 3	44	96	36	37	214
Site 4	67	146	91	97	402
Mean	48	102	45	46	240
Late mature milk					
Site 1	36	73	22	25	156
Site 2	58	106	29	27	219
Site 3	49	81	20	24	173
Site 4	45	124	40	49	259
Mean	47	96	28	31	202
Grand Mean	38	88	31	32	189
SD	13	24	18	20	70
Range	21–67	33–146	19–92	13–97	82–402
American pool[2]	37	70	30	24	161

[1] The data are from 100 individual samples collected at four sites and combined into 16 pooled samples (5–7 individual samples per site per stage of lactation). Site 1, Rouen and Mount Saint Aignau, France; Site 2, Mainz, Germany; Site 3, Bolzano, Italy; Site 4, Treviso, Italy.

[2] A pooled sample of milk collected from 11 American women between 2 and 4 mo postpartum.

TABLE 3
Percentage of total potentially available nucleosides (TPAN) in pooled human milk as adducts, polymeric nucleotides, monomeric nucleotides, and nucleosides[1]

	Uridine	Cytidine	Guanosine	Adenosine	TPAN
			% of total		
Polymeric nucleotides	19 ± 7	57 ± 12	59 ± 21	47 ± 11	48 ± 8
Monomeric nucleotides	36 ± 12	37 ± 13	34 ± 14	35 ± 10	36 ± 10
Nucleosides	18 ± 14	5 ± 5	1 ± 2	5 ± 4	8 ± 6
Adducts[2]	27 ± 12	1 ± 1	7 ± 15	13 ± 9	9 ± 4

[1] $\bar{x}$ ± SD. Based on the mean of the entire pool of human milk collected from 100 individuals at four stages of lactation at four sites.

[2] Adducts are of the form nucleoside-phosphate-phosphate-X, where X is a biologically relevant moiety, for example, uridine diphosphate galactose or NAD.

(36 ± 10%) nucleotide. Nucleosides (8 ± 6%) and nucleotide from adducts (9 ± 4%) were a small but significant contribution. Monomeric and polymeric nucleotides were also the predominant forms of TPAN in the pooled sample of American milk (data not shown) accounting for 93% of the total (excluding adducts).

The distribution of individual nucleotides in each fraction is also shown in Table 3. Uridine was found in all fractions, but primarily as free nucleotide (36 ± 12%) and adduct (27 ± 12%). Cytidine, guanosine, and adenosine were mostly in the polymeric and monomeric nucleotide fractions.

To demonstrate the acceptability of the decision to pool samples, comparisons were made between pooled sample values to the mean values of the five individuals contributing to those pools at site 3 (Italy). The concentrations of individual nucleosides and the TPAN values of the pooled samples and the means of the individuals in the pools at each stage of lactation are given in Table 4. At every stage of lactation, both for individual nucleosides and for TPAN, the measured value of the pooled sample is virtually identical to the mean of the five individual samples that formed that pool.

DISCUSSION

The recovery of adenosine and cytidine from human milk samples (Table 1) appeared to be accurate within 5%. Although the measurement of uridine and guanosine was less accurate, all analyses had high precision. Previous work in this laboratory indicated that the underestimation of guanosine, and probably of uridine, is the result of incomplete elution of these nucleosides from the solid-phase extraction media (boronate derivitized gel). Incomplete enzymatic liberation of the guanosine residues or a contaminating degradative activity that acts on guanosine could also contribute to its underestimation. The TPAN method recovers between 90% and 95% of the true TPAN value, while slightly underestimating the uridine content, and significantly underestimating the guanosine content.

This study answered the questions raised by the preliminary work, and confirmed and extended those data. The failure to detect any increase in TPAN in human milk after cellular disruption by high-intensity sonication indicates that the nucleotide content of the cells present in human milk was accounted for in the TPAN analysis. The procedure by which milk samples were collected, frozen, thawed, and deactivated t ment with strong base apparently resulted in complete c before the analysis.

The addition of nucleotide pyrophosphatase treatme TPAN analysis permitted estimation of the nucleotides from nucleotide-containing adducts. Overall this repre ± 4% of the TPAN value (Table 3), a small but sig addition, because the adducts accounted for a good pc the human milk uridine (27 ± 12%). The work of Ru al (10), Van Buren et al (12), Kulkarni et al (13), and l et al (14) suggests that uridine may account for muc immunological effects attributed to nucleotides.

Most importantly, these data show a wide range of trations of four individual nucleosides as well as the T these 16 pooled samples, representing 100 individual of human milk. Ranges for the entire pool (Table 21–67 μmol uridine/L, 33–146 μmol cytidine/L, 19–9 guanosine/L, and 13–97 μmol adenosine/L, for an range of 82–402 μmol TPAN/L. Cytidine was consiste nucleotide in greatest concentration (88 ± 24 μmol/

TABLE 4
The total potentially available nucleosides (TPAN) of pooled samples compared with individual human milk samples[1]

	Uridine	Cytidine	Guanosine	Adenosine	TPAN
			μmol/L		
Pooled colostrum sample mean	30	82	26	26	164
Mean of samples in pool (n = 5)	29	81	27	27	169
Range of individual samples	17–39	23–144	11–51	17–46	83–253
Pooled transitional milk mean	37	84	43	42	206
Mean of samples in pool (n = 5)	35	85	43	42	214
Range of individual samples	16–61	43–123	10–91	17–86	88–378
Pooled early mature milk mean	44	96	36	37	214
Mean of samples in pool (n = 5)	44	92	35	37	215
Range of individual samples	23–61	61–129	18–80	20–77	126–357
Pooled late mature milk mean	49	81	20	24	173
Mean of samples in pool (n = 5)	47	79	19	23	171
Range of individual samples	23–113	50–108	5–41	6–54	90–325

[1] Pooled samples consisted of individual samples from 20 women (5 per stage of lactation).

MJ. Leach 24.5 45.2 14.1 18.7

MJ. Enfamil sample mg/L

uridine was consistently found in greatest concentrations as adduct (27 ± 12%) and free nucleoside (18 ± 14%). In the comparison between pooled and individual samples from site 3 (Italy), there was excellent agreement between the mean concentrations of the individual samples and the concentration of the pooled sample to which they contributed. However, there was a wide range of values among the samples at all sites over the course of lactation and at any given stage of lactation.

In answer to our third question, this sample-to-sample variability appears to be a property of human milk and not a function of the measurement or stage of lactation. If one assumes that diet varies with nationality, maternal diet also was not a factor. The collection method was well standardized and should not have been a source of variability. Concentrations in colostrum were somewhat lower than the other types of milk, and purine concentrations were generally lower and varied more than the pyrimidine concentrations. However, there was no consistent relation between TPAN concentration, stage of lactation, or country in which the women resided for the European milk samples. The concentration of polymeric and monomeric nucleotides and nucleosides, and the relative proportions of individual nucleosides in the pooled sample of milk from American women were similar to the values in samples of milk from European women (Table 2).

Although there was variability in TPAN among the pooled samples, the percentage of the total contributed by each form was more constant. The nucleotides in these samples were predominantly present as monomeric and polymeric nucleotides. The sum of these two forms ranged from 72% to 92% of the total in the 16 pooled samples with an average of 84%. Consistently, only low concentrations of nucleosides were present, with uridine predominating. Slightly higher concentrations of nucleoside-containing adducts were also present, again with uridine derivatives predominant. Similar relations between the amounts of RNA, nucleotide, and nucleoside were found in the pooled sample of milk from American women (data not shown).

Earlier reports of the nucleotide content of human milk have either described only the monomeric portion or total RNA. Furthermore, previous measurements of RNA in human milk have been less specific or comprehensive. Typical of the nonspecific measurements was a report by Sanguansermsri et al (29) of RNA concentrations in human milk of ≈300–1800 μmol/L. The method of analysis did not involve selective isolation of the nucleic acid fraction before measurement. In addition, the actual measurement procedure was nonspecific and prone to overestimation in complex sample matrixes. More specific and accurate measurements of some forms of ribonucleic acids have been reported. Janas and Picciano (30) measured via HPLC the concentration of mono- and diphosphate nucleotides in human milk during 3 mo of lactation. Gil and Sanchez-Medina (31) reported concentrations for mononucleotides and included many nucleoside-containing adducts (eg, uridine diphosphate hexose and guanosine diphosphate mannose). Neither of these studies of specific forms of nucleotides provided an assessment of the total concentration presumably available in vivo on digestion.

Our data agree well with the previous reports of specific components of the monomeric nucleotide fraction of human milk. When the mean of the 16 pooled samples and the average percentages as nucleotide are used, there are 14 μmol uridine nucleotide/L, 33 μmol cytidine nucleotide/L, 10 μmol guanosine nucleotide/L, and 11 μmol adenosine nucleotide/L for an average total of 68 μmol nucleotides/L and a range of 39–161 μmol nucleotides/L. This measurement does not distinguish between and includes contribution from mono-, di-, and triphosphonucleotides. Janas and Picciano (30) measured mono- and diphosphonucleotide concentrations and reported mean values of 10 μmol uridine nucleotide/L, 27 μmol cytidine nucleotide/L, 6 μmol guanosine nucleotide/L, and 7 μmol adenosine nucleotide/L, for a total of 56 μmol nucleotide/L (including inosine monophosphate).

Janas and Picciano (30) measured inosine monophosphate at various stages of lactation, and the range of their reported values was 1.5–18.4 μmol/L with an average of 6.5 μmol/L. In the present study, inosine derivatives could only be found in 1 of the 16 pooled samples (4 μmol/L in a sample containing 402 μmol TPAN/L) and detected at trace concentrations (> 1 μmol inosine derivative/L milk) in eight others. But 7 of the 16 pooled samples did not contain detectable inosine concentrations. Previous results (data not shown) indicated that human milk contains adenosine deaminase activity (ADA) and that adenosine added to a human milk sample in which ADA had not been deactivated could be partially recovered as inosine. We therefore believe that the presence of inosine in an analysis of human milk may be a sample-preparation artifact. In that regard, the highest concentration of inosine that could be measured represented only 1% of the total of that sample.

Gil and Sanchez-Medina (31) also measured individual nucleotide concentrations, which agree well with this study's determination. In addition, they measured guanosine diphosphate mannose concentrations and found ≈5 μmol/L at various stages of lactation. The average result for adduct-derived guanosine in the present study was ≈2 μmol/L and ranged from not detectable to ≈11 μmol adduct-derived guanosine/L. Gil and Sanchez-Medina (31) also measured uridine diphosphate hexosamine plus uridine diphosphate hexose concentrations, which ranged from ≈5 to > 30 μmol/L. In the present study ≈10 μmol uridine adduct/L was measured, with a range from <1 to ≈21 μmol uridine adduct/L.

There is continued interest among clinical researchers in the field of infant nutrition and some regulatory agencies to accurately determine the amount of ribonucleotides in human milk for use in infant formula. The in vitro enzymatic digestion of the method described here approximates in vivo digestion (Figure 1). Monomeric nucleotides were obtained by the digestion of RNA and nucleoside-containing adducts (a nucleoside-containing adduct is of the general formula nucleoside-phosphate-phosphate-X, where X is any of a group of biologically relevant moieties). Liberated and inherent mononucleotides were further digested to nucleosides, the preferred form for absorption in the gut (3, 7). The subsequent extraction with a boronate derivitized support and separation via HPLC allowed accurate measurement of isolated uridine, cytidine, inosine, guanosine, and adenosine. Measurement of the inherent free nucleosides followed by sequential application of the three enzymatic hydrolyses allowed estimation of the TPAN as polymeric nucleoside (RNA), nucleotide, nucleoside, and nucleoside-containing adduct, and the percentage of each nucleoside present. This complete enzymatic hydrolysis and measurement of the entire nucleotide fraction of human milk is a reasonably accurate reflection of the in vivo process: ie, TPAN.

These data suggest that if there is a need for the addition of nucleotides to infant formula, substantially larger amounts than are currently used would be required to achieve the average TPAN concentration in human milk.

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